Influence of ultrasonic tip distance and orientation on biofilm removal

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Abstract: OBJECTIVE The aim of this study is to assess the effects of ultrasonic tip distance and orientation on the removal of a multispecies biofilm under standardized conditions in vitro. METHODS Six-species biofilms were grown on hydroxyapatite discs for 64 h and treated with a magnetostrictive ultrasonic tip (Cavitron) placed either on contact or at 0.25- and 0.5-mm distance. The treatment was performed for 15 s with either the tip at right angle or sideways. Biofilm removal was evaluated by assessing the viable bacteria in each supernatant and compared to respective controls. In the latter, biofilms were mechanically removed and evaluated in supernatants to assess adhering and floating bacteria. Colony-forming units (CFU) were determined by cultivation on solid media. Any remaining biofilm on the treated discs was also visualized after staining with green-fluorescent SYTO® 9 stain using a confocal laser scanning microscope (CLSM). Mann-Whitney U tests and Bonferroni correction were used to analyze the results between the groups. RESULTS Sideways application of the ultrasonic tip at distances of 0.25 and 0.5 mm removed as many bacteria as present on the control discs compared to the tip on contact ($p < 0.05$). All other application modes, especially the ultrasonic tip applied perpendicularly on contact, showed no statistical significance in removing biofilm. CONCLUSION Overall, data indicated that bacterial detachment depended on tip orientation and distance, especially when the tip was applied sideways similar to the clinical setting. CLINICAL RELEVANCE Biofilm removal by means of ultrasonic debridement remains a crucial aspect in the treatment of periodontal disease. To ensure sufficient biofilm removal, the tip does not necessarily require contact to the surface, but an application parallel to the surface on the side is recommended.

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Influence of ultrasonic tip distance and orientation on biofilm removal

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Abstract

Objectives: To assess the effects of ultrasonic tip distance and orientation on the removal of a multi-species biofilm under standardized conditions *in vitro*.

Methods: Six-species biofilms were grown on hydroxyapatite discs for 64 h and treated with a magnetostrictive ultrasonic tip (Cavitron) placed either on contact or at 0.25 mm and 0.5 mm distance. The treatment was performed for 15 s with either the tip at right angle or sideways. Biofilm removal was evaluated by assessing the viable bacteria in each supernatant and compared to the untreated controls. In the latter, biofilms were mechanically removed and evaluated in supernatants to assess adhering and floating bacteria. CFU were determined by cultivation on solid media. Any remaining biofilm on the treated discs was also visualized after staining with green-fluorescent SYTO® 9 stain using a confocal laser scanning microscope (CLSM). Mann-Whitney-U-Tests and Bonferroni correction was used to analyze the results between the groups.

Results: Sideways application of the ultrasonic tip at distances of 0.25 and 0.5 mm removed as many bacteria as present on the control discs compared to the tip on contact ($p<0.05$). All other application modes, especially the ultrasonic tip applied perpendicularly on contact, showed no statistical significance in removing biofilm.

Conclusion: Overall, data indicated that bacterial detachment depended on tip orientation and distance, especially when the tip was applied sideways similar to the clinical setting.

Clinical relevance: Biofilm removal by means of ultrasonic debridement remains a crucial aspect in the treatment of periodontal disease. To ensure sufficient biofilm removal, the tip does not necessarily require contact to the surface, but an application parallel to the surface on the side is recommended.

Introduction

The treatment of choice in periodontal disease is the removal of subgingival biofilm and pathogens to allow the periodontium to heal [1-3]. This can either be achieved via hand or power driven instruments [4]. Hand instruments have long been in use for the treatment of periodontal disease due to its superior tactile sensation compared to power driven instruments [5]. Nevertheless, later studies could prove same effectiveness of ultrasonic devices in the removal of plaque and calculus as hand instruments with comparable healing response [6-9]. An additional advantage of sonic/ultrasonic debridement is the effective dislodgement of biofilm and deposits as well as residual calculus in areas of limited access, such as furcation lesions, deep vertical defects and multi-rooted teeth [10-13]. Power-driven instruments vary in their design and shape. The insert of a magnetostrictive device for non-surgical supra- and subgingival debridement are usually blunt and dull metal tips with all sides/surfaces being active. The working end of the tip is positioned towards the tooth to remove deposits by means of vibration [14]. Under loaded and unloaded condition, the ultrasonic scaler exhibits oscillation patterns in an elliptical motion [15]. In addition the instrument is run with water for cooling purposes especially at the site of treatment since friction between the scaler and tooth can lead to instant heat formation [16]. Not only cooling of the instrument device, but also irrigation of the site and cleaning properties through biophysical forces were demonstrated. In-vitro-studies show cavitation forces generated through the collapse of water bubbles [17-19]. Simultaneously forces such as acoustic microstreaming in a liquid medium are generated by the ultrasonic tip and decrease with further distance from the area. Acoustic microstreaming cause low-velocity flows and produce high shear stresses [18]. Both acoustic microstreaming and cavitation are related to the amplitude of displacement [18,20,21].

In a previous study, biofilm was removed from HA discs by means of two ultrasonic scalers from different manufacturing companies, one being the piezoelectric miniMaster generator (EMS, Nyon, Switzerland) and the other a Cavitron Select SPS generator (Dentsply, York, PA, USA). In vitro, the magnetostrictive compared to the piezoelectric device was more successful in removing the biofilm from the discs [22]. While both tips under investigations led to bacterial detachment, the action mode as well as the tip configuration and adaptation influenced the biofilm removal potential. One important question remained, however unanswered, namely whether it is possible to remove biofilms without contact of the tips to the surface and how this is influenced by the tip orientation. Hence the objective of this study was to assess the multi-species biofilm removal of the magnetostrictive device orientated in different fixed positions and distances illustrated using a validated in-vitro-model. We hypothesized that it is possible to remove biofilms even in a non-contact treatment approach, but the effectiveness may rather be influenced by the tip orientation and distance:

i) An application on the side of the instrument is more effective than an orientation of the tip perpendicular to the surface and

ii) A short distance from the surface (0.25 mm) leads to more biofilm removal than treatment under contact, but an increasing distance (0.5 mm) may again decrease the effectiveness of biofilm removal.

Material and methods

Biofilm preparation

The specimen used in this study was a multi-species biofilm to simulate natural dental plaque with the most important attribution such as an increased adherence and stickiness. The following description on biofilm preparation was achieved by a standardized and well-published protocol developed in Zurich, Switzerland [23,22]. The six strains used to prepare the biofilms were *Actinomyces oris* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 598, *Streptococcus mutans* OMZ 918, *Streptococcus oralis* OMZ 607 and *Candida albicans* OMZ 110. The biofilms were placed in 24- well polystyrene cell culture plates on HA discs (Ø 9 mm; Clarkson Chromatography Products, South Williamsport, PA, USA) that had been preconditioned (pellicle coated) in 1 ml processed whole unstimulated pooled saliva and incubated for 4 h at room temperature. To initiate biofilm formation, the discs were covered with 1 ml of growth medium containing 30% (v/v) saliva and 70% (v/v) modified fluid universal medium (mFUM), as well as 200 µl microbial suspension prepared from equal volumes and densities of each strain, corresponding to OD_{550}=1.0. The mFUM is a well-established tryptone–yeast-based broth medium designated as FUM [24] and modified by supplementing 67 mM Sorensen's buffer (final pH 7.2). The carbohydrate concentration in mFUM was 0.3 % (w /v), which consisted of glucose for the first 16 h and, from then on of a 1:1 (w/w) mixture of glucose and sucrose. Biofilms were incubated anaerobically at 37 °C for 64 h. After inoculation, the discs remained for 45 min in the growth medium containing 0.3 % glucose and thereafter were subjected to three consecutive 1 min dip washes in 2 ml 0.9 % NaCl to remove growth medium and free floating cells, but not microorganisms adhering firmly to the HA discs. The biofilms were then further incubated in new wells

containing 1 ml of saliva only. After 16 h, 20 h, 24 h, 40 h, 44 h, and 48 h biofilms were pulse-fed by transferring the discs for 45 min into growth medium containing now 0.15 % glucose and 0.15 % sucrose. They were washed again as described above and re-incubated in saliva. Fresh saliva was provided after 16 h and 40 h. After 64 h, the biofilms were dip washed again prior to processing for further treatments and analyses (see below).

Treatment

Biofilms were treated with a magnetostrictive ultrasonic scaler (Cavitron Select SPS generator, Dentsply, York, PA, USA) at medium power setting. Prior to treatment, the HA discs were fixed in Teflon molds and placed in wells of a 24-well cell culture plate. The straight Slimline insert tip was chosen for the Dentsply generator and secured in a fixed position to assure same distances among the runs. The treatments were randomly arranged and each run consisted of three HA discs. The samples were treated for 15 s with the tip of the instrument being perpendicular to the disc followed by the long axis of the instrument being parallel to the disc. Both positions of the instrument were first placed on contact to the disc. Calibrated measuring blocks were used to position the instrument securely in the fixture at 0.25 mm and 0.5 mm distance in each case the tip being perpendicular thereafter the long axis of the instrument being parallel to the disc. In between each treatment, the scaler tip was rinsed with 1.6 ml sterile saline. As previously described, three discs were not treated with the ultrasonic scaler, but biofilms were manually scraped off the surface to determine the total colony-forming units (CFU). Two of the three discs were used to analyze the remaining biofilms on the disc, while the third randomly selected disc was used to analyze the remaining biofilm using CLSM. Three independent

experiments were performed, and within each experiment every group was represented in triplicate biofilm cultures, resulting in N=9.

Analysis of biofilm removal

The removal of biofilm and its analysis followed the same protocol as previously described [22]. In brief, non- adherent bacteria, present in the supernatant were collected to determine the amount of bacteria that fell into solution and were subtracted from the results of the treatment runs (“control supernatant” in Fig. 1). The amount of potentially growing bacteria without ultrasonic treatment was determined by culture analyses after manually scraping (Perio Soft-Scaler, Kerr, Bioggio, Switzerland) biofilms off the discs and rinsing the latter with 1.6 ml sterile saline to remove non- adherent bacteria. Serial dilutions of suspended biofilm bacteria were prepared in 0.9 % NaCl, and 50-µl aliquots were plated on Columbia blood agar supplemented with 5 % whole human blood to estimate total CFU. Agar plates were incubated anaerobically at 37 °C for 72 h. After treatment with the ultrasonic scaler at various positions and distances, the solution of the supernatant was collected (1.6 ml), and determination of total CFU of the supernatant was done as described above (Fig. 1). Data were scored as total CFU per biofilm. All microbiological tests and analyses were performed strictly blinded to the nature of the previous treatment of the individual discs.

Staining of biofilms and CLSM

For CLSM, treated and untreated biofilms were stained using Syto 9 green

fluorescent nucleic acid stain (Invitrogen, Zug, Switzerland) according to the instructions of the manufacturer. After 20 min of staining, excess dye was gently aspirated from the discs without touching the biofilms. They were embedded upside down in 20 µl of Mowiol [25], stored at room temperature in the dark for at least 6 h prior to CLSM examination.

Stained biofilms were examined by CLSM using a Leica TCS SP5 (Leica Microsystems, Heidelberg GmbH, Germany) with a ×20/0.8 numerical aperture (NA) oil immersion objective lens in conjunction with 488-nm laser excitation and 530-nm emission filters for Syto 9. Image acquisition was done in x6 line average mode. Scans were recombined and processed using Imaris 7.6.5 (Bitplane AG, Zurich, Switzerland)

Statistical analysis

Statistical analysis was performed with StatView Version 4.51 (Abacus Concepts Inc., Berkeley, California, USA) and represented in a box plots. The Mann-Whitney-U-Tests and Bonferroni correction was used to analyze the results between the groups.

Results

In order to evaluate the removal of biofilms during each procedure the bacterial CFU in the culture supernatants were counted and compared to the control. Figure 2 illustrates the capacity of biofilm removal of each procedure starting with the untreated control. The CFU of the control disc represents the maximum amount of removable biofilm. The CFU of the “control supernatant” displays the amount of spontaneously detached biofilm, which were part of the calculations on the treatment groups. Compared to the control discs, the contact treatments as well as the non-contact tip treatment showed statistically significant differences of CFU, i.e. that the amount of removed biofilm was lower than in the control experiment. Only the side application parallel to the disc with a distance of 0.25 mm and 0.5 mm did not show significant results compared to the control which means that these treatments removed close to as many bacteria as present on the control disc.

Figure 3 displays an illustrative series of CLSM images of the biofilms on the HA surfaces before and after treatment. The images were taken diagonally along the diameter (9 mm) of the discs at a distance of 5 µm from the HA surface, thus, showing only a cutout of the biofilm on the disc. The CLMS images of the biofilms confirm the results described above for CFU analysis. Slides 3 a and 3 b show the control discs one being the untreated and the other being the scraped clean disc. The least effective treatment results (Fig. 3 c-e) were seen with the tip of the instrument placed perpendicular to the disc in chronological order from contact up to 0.5 mm distance. The most effective treatment appeared to be the application of the tip parallel to the disc. The removal of biofilm at a distance of 0.25 and 0.5 mm (Fig. 3 g-h), respectively, showed a clean surface, which was comparable to the control (Fig. 3 b) using a scaler to manually remove the biofilms.

Discussion

This study investigated the biofilm removal capacity of a magnetostrictive ultrasonic tip when orientated in different positions and distances. To determine the biofilm removal potential, the CFU of bacteria in the supernatant were analyzed using a conventional culture technique. Additionally, CLSM images were made to visualize the biofilms. Both methods confirmed that the best results were achieved when the ultrasonic tip was placed parallel to the HA disc corroborating the results of a previous study using magnetostrictive and piezoelectric devices, which assessed the tip orientation [22]. At a distance of 0.5 mm and a parallel application of the instrument, however, no significant difference of biofilm removal compared to the values obtained at 0.25 mm was observed.

Several studies have been undertaken to assess different chemical and physical operating conditions, which help explain the ultrasonic cleaning efficacy. In principle, Acoustic cavitational activity induces gas bubbles in aqueous solutions, which burst and release free radicals near the surface [26]. An additional phenomenon observed are the acoustic microstreaming forces, referring to the formation of radial and tangential stresses, shearing off plaque at a rapid velocity and help aid in the cleaning mechanism [26,18]. Nevertheless, the entire cleaning mechanism of power driven devices is not fully understood.

Additional factors such as different power settings, instrument designs and increased operational times may also contribute to the cleaning success and performance of the ultrasonic device [27-29]. In the present study we have chosen to use a medium power level as recommended by Flemmig and co-workers [30,31]. The selection of biofilm preparation, instrument tip and time frame stood in correspondence to a previous study done by Thurnheer et al. [22]. The straight Slimline insert tip for the

Dentsply generator showed significant better results to remove biofilm. Also the application of the tip placed parallel to the side showed more favorable outcomes. Therefore, this instrument type was chosen in the present set-up. The instrument design is a crucial factor in the formation of cavitation to occur under loaded conditions, referring to the development of increased vibration displacement amplitude at the tip. One study investigated the occurrence of cavitation on different ultrasonic tips [32]. Under loaded condition via teeth in a measuring apparatus the displacement and cavitational production towards the tip was in favor of a thinner and longer scaler probe. On the contrary to the expected increased vibration amplitude, the present study showed no significant results to the supernatant with the tip of the instrument placed perpendicular to the disc when being in contact. Little dislodgment was also seen on the CLSM images. Referring to the previous work of Thurnheer et al. suggesting the embedding and attachment of the samples could influence the vibration transduction and result in a different outcome [22].

As hypothesized, the instrument tip placed perpendicularly to the surface at an increased distance was less efficient in removing biofilms. A previous study by Felver and co-workers examined cavitation activity around ultrasonic tips [33]. The end of the tip displayed the highest degree of motion but, surprisingly the maximum amount of cavitation did not occur at the end of the tip rather along the tip towards the bend region of the instruments. Khambay & Walmsley investigated the effect of the acoustic microstreaming on particulate matter as a substitute for plaque [18]. Similar findings on the orientation of the tip as well as the distance of the ultrasonic tip could be found. An application with the tip on the side parallel to the disc as well as with increased distance of the tip towards the slide showed the best results in the removal of the used surrogate material in their study.

Cleaning efficacy by means of non-contact mechanisms has also been the focus in studies with power driven toothbrushes. Busscher and co-workers evaluated biofilm removal of oscillating-rotating and sonic toothbrushes at a distance up to 6 mm [34]. Biofilm removal could effectively be demonstrated through hydrodynamic transfer, air-bubble inclusion and acoustic energy. However, with increased distance a decrease of biofilm disruption was still observed, but a 100% biofilm removal could never be achieved.

An additional question raised by a systematic review was the area of interdental cleaning efficacy with power driven toothbrushes. Hence, the study focused on the current in vitro evidence regarding power driven toothbrushes in relation to biofilm removal under a noncontact setting [35]. Most of the sixteen studies included reported on a biofilm removal, exceeding 50%.

In accordance with previous studies streaming forces in liquid medium, displacement amplitude of the instrument tip and cavitational activity are required to remove debris [20,32]. As seen in the CLSM images, it appears that the greater the distance of the instrument tip placed parallel to the surface the greater the amplitude for displacement hence being able to dislodge a broader surface with higher efficacy. In contrast, the tip tilted perpendicular to the surface only showed a small area of cleaned surface on the slide. The most and broadest removal in this setting was seen with the tip being in contact with the disc compared to a distance of 0.25 mm. Almost no effect could be observed when the tip was placed at a distance of 0.5 mm.

During the course of supportive periodontal maintenance therapy with numerous debridement sessions over the years, potential damage to the root gains an important aspect as the cumulative effect of consecutive dentin removal has been a proven fact for hand instruments. Although there has been less damage on root

surfaces due to ultrasonic scaling devices reported, studies suggest some degree of surface alteration due to ultrasonic instrumentation. Jepsen and co-workers investigated the loss of root dentin in vitro, caused by different magnetostrictive and piezoelectric ultrasonic insert tips [36]. Again, dentin alterations showed to be significantly influenced by instrument design and width of the tip. An increase of aggressiveness to the root surface was primarily seen in wider scaler tips as compared to narrow tips and secondly increased with an application force from 0.3 N to 0.7 N. The present study used a medium power level setting of the magnetostrictive device for each run and no defects or alterations on the surface of the discs could be observed. However, this study cannot exclude potential microscopic defects on the discs, especially since a previous study on magnetostrictive ultrasonic instruments at different power settings proved a significant change in dentinal roughness and loss of dentin with increased power, starting with at a medium setting [14]. Further investigational studies are required to render a significant proposition for clinicians as this was not the focus of this investigation. Lea & Walmsley released proposals for future standardized investigations on all ultrasonic devices in order to enable comparisons between studies regarding efficacy, power settings, surface alteration or defects to name only a view examples [37].

In summary, several *in vitro* studies on ultrasonic scalers could prove their efficacy in biofilm removal. Under these controlled circumstance there have been multiple biofilm models developed to mimic the complex oral environment under clinical conditions [38-42]. This study utilized a six species biofilm which represents gingival plaque. The biofilm model has been validated and is well established on different materials. Nevertheless clinical success is only determined through gingival pocket

depth reduction and absence of bleeding. The completion of biofilm removal in a patient's mouth is not necessary the determining factor for success and absence of disease. However, further research is still needed to determine the true mechanism behind the removal of biofilm with ultrasonic scalers. Various operational conditions such as instrument design, power settings, operation time, instrument placement and distance have shown to aid in cleaning success since studies on displacement amplitude may not be the only cause of cavitation formation[32].

Conclusion

With regard to the research objective the instrument tip placed parallel to the surface at a distance of 0.25 mm and 0.5 mm these settings were most effective in removing biofilms. This is of clinical relevance since the action mode of the ultrasonic scaler is not due to the amount of force used rather due to the mechanical motion of the instrument itself and the ultrasonic effects.

Captions

Fig. 1

Flowchart of the different steps in preparing the analysis of biofilm removal.

Fig. 2

Box-plot presentation of the CFU counts in the experimental and control group. Identical capital letters show a significantly statistical difference in treatment performed in contrast to the control disc. Different capital letters show no significantly statistical difference to the control disc, signaling nearly the same amount of biofilms present on the control disc were removed during the treatment.

Fig. 3

CLSM images of in-vitro-biofilms on the HA surface untreated and after treatment. Images were taken along the diameter of the discs (Ø = 9 mm) at 5 µm above the HA surface and represent only a detail of the whole disc. The biofilms were stained using green-fluorescent SYTO® 9 DNA stain.

Compliance with ethical standards

Conflict of interest

All authors declare that they have no competing interests.

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Ethical approval

All procedures performed in the present study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Since the samples were irreversibly anonymized, the study complied with the local ethics guidelines and did not require an individual ethics approval (Swiss Federal Council, Federal Act on Research involving Human Beings).

Informed consent

All participants gave their informed consent.

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Fig. 1

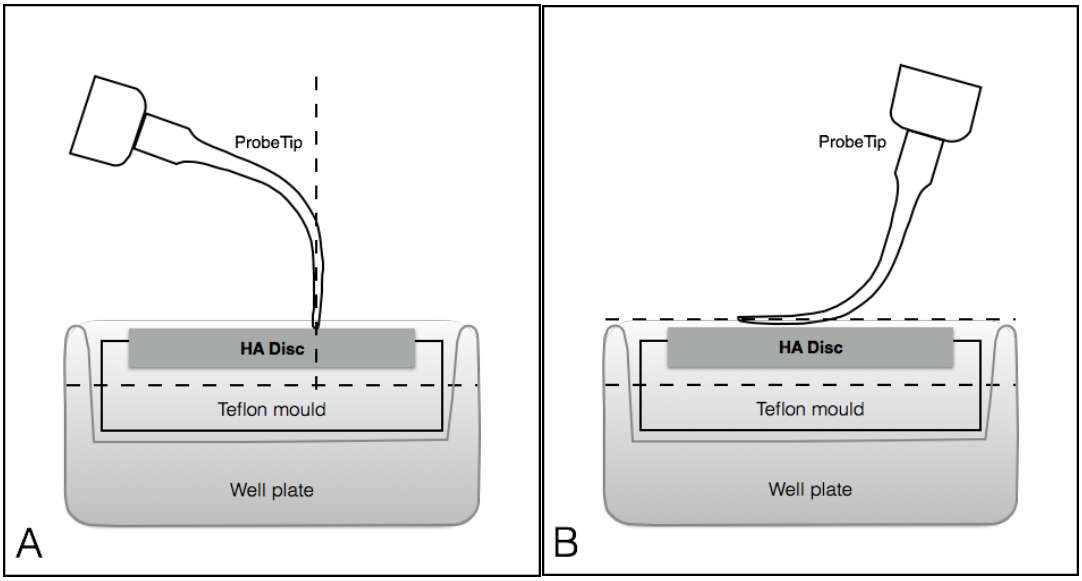

ProbeTip
HA Disc
Teflon mould
Well plate
A
ProbeTip
HA Disc
Teflon mould
Well plate
B

Fig. 2

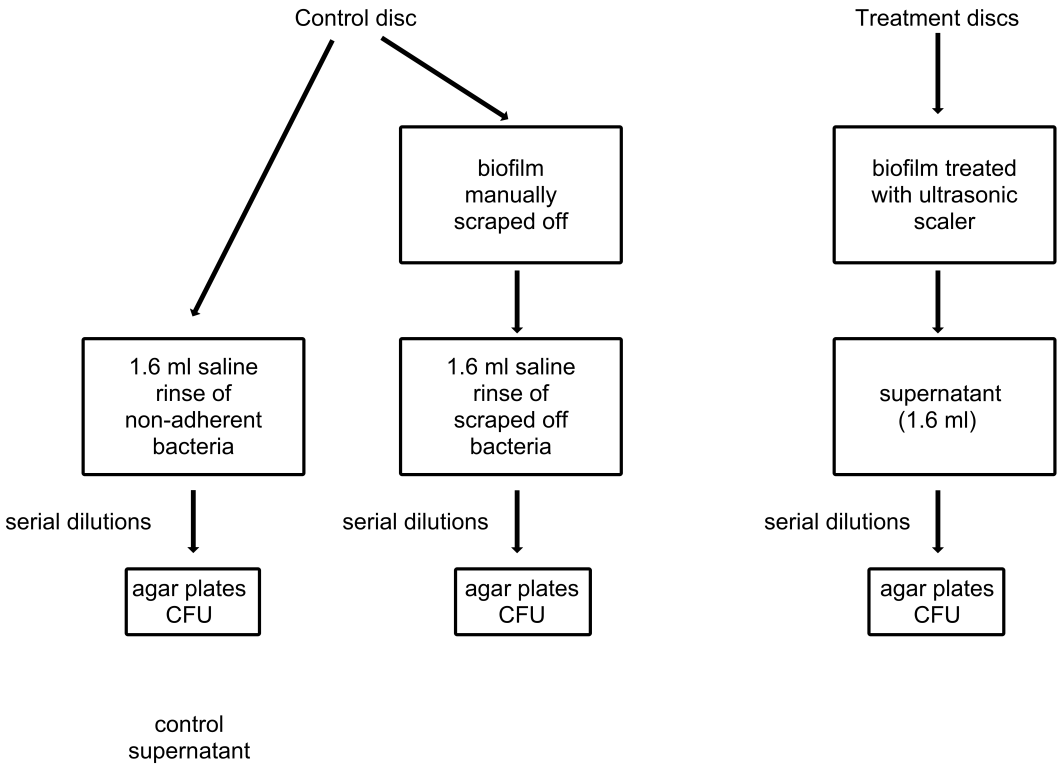

Control disc
Treatment discs
biofilm manually scraped off
biofilm treated with ultrasonic scaler
1.6 ml saline rinse of non-adherent bacteria
1.6 ml saline rinse of scraped off bacteria
supernatant (1.6 ml)
serial dilutions
serial dilutions
serial dilutions
agar plates CFU
agar plates CFU
agar plates CFU
control supernatant

Fig. 3

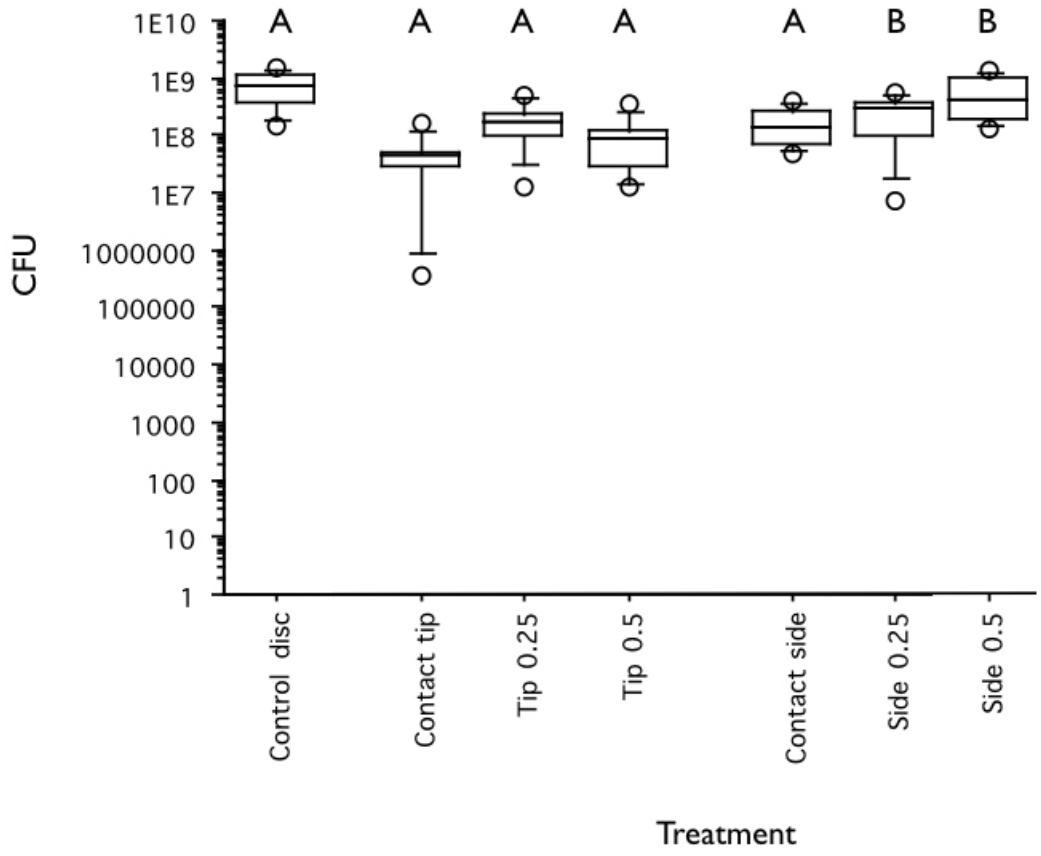
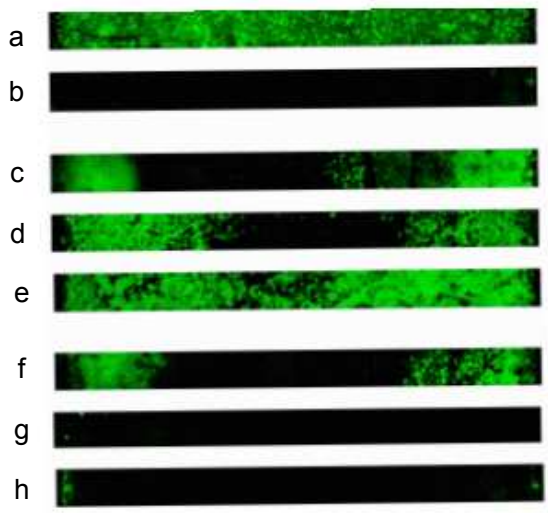

A
A
A
A
A
B
B
1E10
1E9
1E8
1E7
1000000
100000
10000
1000
100
10
1
CFU
Control disc
Contact tip
Tip 0.25
Tip 0.5
Contact side
Side 0.25
Side 0.5
Treatment

Figure 4



Control disc (before scaling)
Control disc (manual scaling)

Contact tip
Tip 0.25 mm
Tip 0.5 mm

Contact side
Side 0.25 mm
Side 0.5 mm